EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

ECBC-TR-452

LOW-LEVEL CYCLO-SARIN (GF) VAPOR EXPOSURE IN THE GOTTINGEN MINIPIG: EFFECT OF EXPOSURE CONCENTRATION AND DURATION ON PUPIL SIZE

Stanley W. Hulet
Douglas R. Sommerville
Bernard J. Benton
Jeffry S. Forster
Jacqueline A. Scotto
William T. Muse
Ronald B. Crosier
Sharon A. Reutter
Robert J. Mioduszewski
Sandra A. Thomson

RESEARCH AND TECHNOLOGY DIRECTORATE

Dennis B. Miller
Jill R. Jarvis
Candice L. Krauthauser

SAIC

SCIENCE APPLICATIONS
INTERNATIONAL CORPORATION
Abingdon, MD 21009

May 2007

ABERDEEN PROVING GROUND, MD 21010-5424

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-05-2007	Final	Mar 04 - Sep 04

4. TITLE AND SUBTITLE
Low-Level Cyclo-Sarin (GF) Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hulet, Stanley W.; Sommerville, Douglas R.; Benton, Bernard J.; Forster, Jeffry S.; Scotto, Jacqueline A.; Muse, William T.; Crosier, Ronald B.; Reutter, Sharon A.; Mioduszewski, Robert J.; Thomson, Sandra A. (ECBC); Miller, Dennis B.*; Jarvis, Jill R.*; Krauthauser, Candice L.* (SAIC)

5d. PROJECT NUMBER
201400

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
DIR, ECBC, ATTN: AMSRD-ECB-RT-TT, APG, MD 21010-5424
Science Applications International Corporation (SAIC), 3465A Box Hill Corporate Drive, Abingdon, MD 21009

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-452

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency, 8725 John J. Kingman Road, MS 6201, Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
*When the work was conducted, the authors indicated above were employed by Geo-Centers, Inc., which is now part of SAIC.

14. ABSTRACT

The present study utilized infrared pupillometry to digitally capture images of pupils in real-time during whole-body vapor GF exposures of Gottingen minipigs. Binary normal regression was used to fit various response models to the data. Values for EC_{50} and ECT_{50} were calculated for miosis in male and female minipigs exposed to GF vapor for 10, 60, and 180 min. There is a 32% overall difference (area basis) in the model fits for the miotic ECT_{50} values between the genders, with the males being more sensitive. The difference between the genders became more pronounced at the longer exposure-durations. The ECT_{50} associated with miosis was not constant over time as predicted by Haber's rule. Rather, the data were best described by a toxic load model. The value of the best model fit for the toxic load exponent was 1.60 with 95% confidence interval of 1.38 to 1.82. The best estimate of the probit slope (concentration) was 12.4 with 95% confidence interval of 4.8 to 20.0. Potential curvature in the data with respect to fitting by the toxic load model was evaluated by inserting the term, $(LogT)^2$, and this term was found to be statistically insignificant ($p > 0.3$).

15. SUBJECT TERMS

Toxic load model	Probit slope	Concentration	Inhalation	Cyclo-sarin	Gottingen
Duration	Miosis	Low level	GF	Minipig	Swine
Ct	EC_{50}	ECT_{50}	$(LogT)^3$	($p > 0.3$)	

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Sandra J. Johnson
U	U	U	UL	49	19b. TELEPHONE NUMBER *(include area code)* (410) 436-2914

Blank

QUALITY ASSURANCE

This study, conducted as described in Protocol 02-341, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 17 Aug 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase Inspected	Date	Reported
Study parameters and exposure	26 Sep 02	26 Sep 02
	06 Feb 03	06 Feb 03
Data and Final Report	28 Jul 04	28 Jul 04

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

DENNIS W. JOHNSON
Quality Assurance Coordinator
Toxicology, Aerosol Sciences and
Obscurants Senior Team
Research and Technology Dir.

Blank

PREFACE

The work described in this report was authorized under Project No. 201400, Low-level Toxicology. The work was started in March of 2004 and completed in September of 2004. The experimental data is contained in laboratory notebook 04-0002 and on compact discs. Raw data and the final report from this study are stored in the Toxicology Archives, Building E-3150, Aberdeen Proving Ground, MD.

In conducting this study, investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23, 1985, as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, Washington, D.C. These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care and Public Affairs," and the U.S. Army Edgewood Chemical Biological Center (ECBC) Institutional Animal Care and Use Committee (IACUC), which oversees the use of laboratory animals. This project's assigned IACUC protocol No. 02-341 was approved on 6 August 2002.

This report has been approved for public release. Registered users should request additional copies from the Defense Technical Information Center; unregistered users should direct such requests to the National Technical Information Service.

Acknowledgments

The authors thank Dr. Julie Watson and Dr. Diana Scorpio (SAIC) for their support in caring for and handling the animals used in this study. The authors also thank Dennis Johnson (Veterinary Services Team, ECBC) for Quality Assurance assistance.

Blank

CONTENTS

FIGURES

TABLES

LOW-LEVEL CYCLO-SARIN (GF) VAPOR EXPOSURE IN THE GOTTINGEN MINIPIG: EFFECT OF EXPOSURE CONCENTRATION AND DURATION ON PUPIL SIZE

1. INTRODUCTION

The primary objective of the current research study is to understand thoroughly the dose-response relationship between traditional chemical warfare (CW) nerve agents and living species. Constriction of the pupil (miosis) is often the first noticeable effect of a vapor exposure, thereby making it an ideal biological endpoint for determining and modeling threshold dose-response relationships. Pupil constriction induced by nerve agents is thought to be a local effect caused by direct contact of the nerve agent with the eye.[1] Local inhibition of cholinesterase results in excessive stimulation of muscarinic receptors at the pupillary sphincter muscles of the iris and the ciliary muscle of the lens resulting in pupil constriction and problems with accommodation. The inability of the pupil to dilate can also result in loss of dark adaptation.[2] Given that military operations are often conducted at night, determining the threshold levels for nerve agent intoxication in dim-light situations is vital.

Gauging the biological impact of nerve agent vapor exposure on the eye is necessary to quantitatively relate the probability of eye responses to appropriate exposure parameters. Traditionally, inhalation and ocular toxicology have used dosage (as expressed by the product of exposure concentration (C) and exposure-duration (T)) as a metric of toxicant exposure.[3] The time dependence of CW agent toxicity has been modeled using Haber's rule, which assumes that ECT_{50} is constant with the value of T.[4] However, this concept has been found to be inadequate for assessing biological effects from exposure to many acutely toxic gases and aerosols.[5]

In the current study, whole-body inhalation exposures with Cyclohexyl methylphosphonofluoridate (GF or cyclo-sarin) were performed on Gottingen minipigs. A thorough review of the limited data available on GF exposures has been presented by Whalley *et al.*[6] who point out the lack of experimental data investigating low-level toxicity via the inhalation exposure route. Indeed, Whalley and his colleagues were the first to investigate pupil constriction resulting from non-lethal, low-level exposure to GF. They have defined ECT_{50} (miosis) values for vapor GF exposures of 10, 60, and 240 min in rats. The data are best fit using a toxic load model[5] as opposed to Haber's rule. In the toxic-load model, dosage is not used to quantify the amount of toxic material received. Instead, a new term, toxic load (TL), has been developed and extensively used, with TL equaling C^nT.[7] The TL exponent, n, is dependent upon the toxicant and exposure scenario. The median effective dosage (ECT_{50}) no longer remains constant but becomes dependent on exposure time. A comparison between the TL model and Haber's rule is shown in Figure 1. Whalley's study provided the first evidence that when pupil constriction is used as the biological endpoint and GF is the nerve agent, the results of low-level vapor exposure are not constant over time. Whalley's data support similar conclusions reached from low-level sarin (GB) exposures in rats[8] and minipigs.[9]

A comprehensive study comparing GB and GF potencies in rats, exposed to potentially lethal vapor concentrations of the two agents, provides indisputable evidence that potency ratio changes drastically with the value of T.[10] The two agents were relatively equipotent for a 10-min exposure, but when T was increased to 240 min, GF became almost half as potent as GB. Potency comparisons between GB and GF at low-level vapor concentrations cannot be successfully drawn because of a lack of data. Two separate studies with the two agents have been conducted using rats. The first determined ECT_{50} values for GF vapor exposure on pupil constriction[6] and the second ascertained ECT_{50} values for GB vapor exposure.[8] However, as different methods of data collection were used in each, no viable comparative conclusions could be reached for low-level GF and GB potencies.

The current study with minipigs exposed to GF complements the work described by Hulet *et al.* (2006) with GB.[9] By using the same method of pupil assessment in the same species, a viable comparison between the potencies of the two agents could be drawn.

Many anatomic and physiologic similarities exist between pigs and humans.[11] The current study estimated effective (miosis) concentrations of the nerve agent cyclo-sarin (GF) as a function of T in the Gottingen minipig and also determined dependency of the median effective dosage (ECT_{50}) on exposure time.

2. MATERIALS AND METHODS

2.1 Animals.

Male and female Gottingen minipigs were obtained from Marshall Farms USA (North Rose, NY). Upon arrival at the facility, the minipigs underwent an initial health examination by the attending veterinary staff. The pigs were then quarantined for a minimum of three days. After this time, the research personnel familiarized the pigs to various procedures that included daily handling, change of location within the animal facilities, and a sling apparatus. While the pigs were in their cages, they were periodically enriched by human interaction and given unfettered access to play toys (hanging chains, bunny balls) and food treats.

2.2 Surgeries.

A silicone catheter was surgically implanted in the external jugular vein of each minipig.[9] Each surgical site (lateral neck from mandible to shoulder and mid dorsally between the shoulder blades) was prepared for aseptic surgery by close-clipping the area and applying a surgical scrub (chlorhexidine or povidone-iodine) followed by an application of isopropyl alcohol or sporicidal agent.

Each catheter (Bard access systems, 6.6 or 9.6 Fr.), impregnated with heparin and antimicrobial agent, was first inserted into an external jugular vein of the pig and then advanced to the anterior vena cava or right atrium. A subcutaneous tunnel, extending from the surgical site adjacent to the jugular vein to the exit site in the dorsal midline, was created with a hollow stainless-steel rod. The catheter was filled with sterile heparin saline (1%), grasped and pulled

through from the dorsum to the ventral neck incision with at least 6 in. of catheter remaining external to the skin. The catheter position was adjusted so that blood samples could be readily obtained. The catheter was secured into the vein by tying at least two sutures around the vein. A loop of the catheter leading from the vein was secured to the subcutaneous tissues using sutures. Once the catheter was properly adjusted, it was secured at the dorsal exit site and the incisions closed. The catheter was locked with 1% sterile heparin saline. Triple Antibiotic ointment was placed on both incisions. Postoperatively, each pig was administered analgesics (buprenorphine 0.01 - 0.05mg/kg, BD) for at least 24 hr and subsequently, if needed.

The pigs were allowed at least three days for recovery from the surgical implantation of the indwelling catheter before they were exposed to the nerve agent vapor. During that time, each catheter vascular access port was flushed with heparinized saline, as needed. During the agent exposures, the catheters were maintained by a continuous intravenous infusion of lactated Ringers solution. Blood samples were taken from the pigs periodically for assessing cholinesterase inhibition and internal agent levels via GF regeneration assays.[12]

2.3 Sling Restraint.

A sling was used to restrain the minipigs during exposure. The frame of the sling was constructed of airtight stainless steel pipe and Swagelok™ fittings. The slings were custom designed (Lomir Biomedical, Inc., Malone, NY or Canvas and Awning supplies, White Marsh, MD) to fit the frame and the size of the pigs used in the studies. Each sling was constructed of canvas with four-leg holes so that the canvas fitted comfortably around the pig's belly. Two straps secured over the pig's shoulders and hips, and a muzzle harness was placed over the pig's snout and secured both laterally and ventrally to the stainless-steel framing to prevent free movement of the head. This way, a constant angle and distance (40 in.) from the infrared (IR) camera to the pig's eye could be maintained. The harness did not interfere with the pig's ability to breathe.

2.4 Inhalation Chamber.

Whole-body minipig exposures were conducted in a 1000-liter dynamic airflow inhalation chamber. The Rochester style chamber was constructed of stainless steel with glass or Plexiglas windows on each of its six sides. The interior air was maintained under negative pressure (0.25-0.30" H_2O), which was monitored with a calibrated magnehelix (Dwyer, Michigan City, IN). A thermoanemometer (Model 8565, Alnor, Skokie, IL) was used to monitor chamber airflow from the outlet.

Two sampling methods were used to monitor and analyze the GF vapor concentration in the exposure chamber. The first method was a quantitative technique using solid sorbent tubes (Tenax/TA) to trap GF, followed by thermal desorption and gas chromatographic (GC) analysis (HP Model 6890, Agilent Technology, Baltimore, MD). The second method was a continuous monitoring technique using a phosphorus monitor (HYFED, Model PA260 or PH262, Columbia Scientific, Austin, Texas). Output from the HYFED provided a continuous strip chart record of the rise, equilibrium, and decay of the chamber vapor concentration during an exposure.

All samples were drawn from the middle of the chamber. Solid sorbent tube samples were drawn after the chamber attained equilibrium (t_{99}), while the HYFED monitored the entire run. Solid sorbent tube samples were drawn from the chamber approximately every 10 min with each sample draw lasting 1-5 min depending upon chamber vapor concentration and duration of exposure. All sample flow rates for the solid sorbent tube systems were controlled with calibrated mass flow controllers (Matheson Gas Products, Montgomeryville, PA). Flow rates were verified before and after sampling by temporarily connecting a calibrated flow meter (DryCal®, Bios International, Pompton Plains, NJ) in-line to the sample stream. Physical parameters (chamber airflow, chamber room temperature, and relative humidity) were monitored during each exposure and recorded periodically.

2.5 IR Camera.

A Sony CCD black and white video camera (model XC-ST50), equipped with 2-IR 100-candlepower spotlights, was focused on the left pupil of a minipig for the duration of the nerve agent exposure. The distance between the camera and the eye was standardized at approximately 40 in. and the images were shot through the external Plexiglas of the exposure chamber at a consistent angle. Plexiglas does not interfere with the quality of IR images.

Sequential images of the eye, under dim light conditions, were digitally captured for the analysis and calculation of the pupil area. The GF exposures were for 10, 60, or 180 min. However, the minipigs were required to remain in the exposure chambers for an additional 15 min for out-gassing. The pigs were then removed from the chambers and additional images were captured for the next 50 to 60 min to ensure there was no further decrease in pupil area.

2.6 IR Pupillometry.

The basis of IR pupillometry is the reflection of IR light off the retina and back through the pupil to the camera producing a bright pupil image. This method uses an IR light that causes no constriction and allows pupil area measurements to be calculated under dim light conditions. The IR method maximizes pupil area and provides for measurements in a realistic environment. Real-time images of pig pupils are captured during exposure and saved for quantifying pupil area off-line.[9] The images are captured, filtered and quantified using a custom designed software program.[13]

If a minipig showed 50% reduction in pupil area at any time during the sarin exposure or observation period following exposure, it was classified "positive" for miosis.

2.7 Solid Sorbent Tube System.

The automated solid sorbent tube sampling system consisted of four parts:

(1) A heated sample transfer line
(2) A heated external switching valve
(3) A thermal desorption unit
(4) A gas chromatograph.

A steel sample line (1/16 in. o.d. x 0.004 in. i.d. x 6 ft. length) extended from the middle of the chamber to an external sample valve. The sample line was commercially treated with a silica coating (Silicasteel® Restek, Bellefonte, PA) and covered with a heated (60 °C) sample transfer line (CMS, Birmingham, AL). The combination of line coating and heating minimized nerve agent adhesion to the sample line interior. From the transfer line, the sample entered a heated (125 °C) 6-port gas-switching valve (UWP, Valco Instruments, Houston, TX). In the by-pass mode, vapor from the chamber continuously purged through the sample line to a charcoal filter. In the sample mode, the gas sample valve redirected nerve agent vapors from the sample line to a Tenax TA/Haysep sorbent tube (60-80 mesh) located in the thermal desorption unit (ACEM-900, Dynatherm Analytical Instruments, Kelton, PA). Temperature and flow programming within the Dynatherm desorbed nerve agents from the sorbent tube directly onto the GC column (RTX-5, 30 m, 0.32 mm i.d., 1 mm thickness), which was then followed by flame photometric detection (FPD-phosphorus mode).

The solid sorbent tube sampling system was calibrated by direct injection of external standards (GF μg/mL) into the heated sample line of the Dynatherm. This way, injected nerve agent standards were put through the same sampling and analysis stream as the chamber samples. A linear regression fit ($r^2 = 0.999$) of the standard data was used to compute the GF concentration of each chamber sample.

2.8 Chemicals.

Cyclohexyl methylphosphonofluoridate (GF or cyclo-sarin) was used for all the vapor exposures in this study. The munitions grade GF (lot # GF-93-0034-147.2) was verified as 98.16 ± 0.36 wt.% pure as determined by quantitative ^{31}P-NMR and stored in sealed ampoules containing nitrogen. Ampoules were opened as needed to prepare external standards or to be used as neat agent for vapor generation. All external standards for GF vapor quantification were prepared on a daily basis. Triethylphosphate (99.9% purity), obtained from Aldrich Chemicals (Milwaukee, WI) was used as the internal standard for the GF purity assays.

Analysis for agent impurities was conducted using acid-base titration, Gas Chromatography/Mass spectrometry (GC-MS), and ^{1}H NMR. Acid-base titration has been found to show the following impurity percentages based on mole ratios:

GF ANALYSIS

Compound	Calculated Wt %
GF Acid	1.51 ± 0.13

2.9 Vapor Generation.

Saturated GF vapor streams were generated by forcing nitrogen carrier gas (N_2) through a glass vessel (multi-pass saturator cell) that contained liquid GF. The saturator cell consisted of a 100-mm long, 25-mm o.d. cylindrical glass tube with two (inlet, outlet) vertical 7-mm o.d. tubes connected at each end. The main body of the saturator cell contained a hollow ceramic cylinder that served to increase the contact area between the liquid nerve agent and the N_2. The saturator cell was fabricated to allow N_2 to make three passes along the surface of the

wetted ceramic cylinder (Alundum® Fused Alumina, Norton Co., Colorado Springs, CO) before exiting the outlet arm of the glass cell. The saturator cell body was immersed in a constant temperature bath so that a combination of N_2 flow and temperature could regulate the amount of nerve agent vapor entering into the inhalation chamber. The entire apparatus was contained within a generator box mounted at the top of the inhalation chamber. Typically, the saturator cell was loaded with 2-3 mL of liquid GF. To maintain the integrity of the liquid nerve agent within the cell, a continuous low flow rate (5-10 mL/min) N_2 stream was used.

2.10 Blood Sample Collection.

The indwelling jugular catheters implanted in the pigs enabled the draw of "real time" blood samples for assessing cholinesterase inhibition and internal agent levels via GF regeneration assays.[12] Blood was drawn from the pigs just prior to exposure and at periodic intervals throughout exposure: approximately every 2 min during the 10-min exposure, every 15 min during the 60-min exposure, and every 30 min during the 180-min exposure. The total volume of blood drawn did not exceed 1% of the minipig's body weight over a 1-week span. The drawn, sample volume of blood was replaced by an equivalent volume of Lactated Ringers.

2.11 Design and Data Analysis.

The up and down method was used with an assumed probit slope of 10[14] to determine the progression of experimental exposure concentrations. The binary response used for executing the up and down method was the presence or absence of miosis.

The method of maximum likelihood estimation (MLE)[15] was used on the resulting quantal data to calculate ECT_{50} (miosis) values, with approximate 95% confidence intervals, for each of the six gender exposure-duration groups. The MLE calculations were also performed on a pupil diameter basis-the shorter axis of the pig's elliptical pupil was used as the diameter because the shorter axis goes to zero at complete constriction. These analyses used the assumed probit slope of 10. An example of an MLE calculation has been shown by Hulet *et al.*[9]

Ordinarily, the number of animals used in an experiment using the up and down method is not enough to permit reliable estimation of the probit slope. However, data from several up and down experiments can be combined to form a subject pool large enough to estimate the probit slope,[9] which can be analyzed by traditional probit analysis[16] or by binary logistic or normal regression.[17] A TL model is fit to the combined data (from the six up and down experiments) by binary regression with a normit link function (using the binary logistic routine with a normit link function in MINITAB® --see below).

Equations 1 and 2 were used for modeling the probability of miosis:

$$Y_N = (Y_P - 5) = k_0 + k_C(\log_{10} C) + \sum_{T}^{3}\sum_{S}^{2} k_{T,S}\,(Time)_T\,(Gender)_S \qquad (1)$$

$$Y_N = (Y_P - 5) = k_0 + k_C(\log_{10} C) + k_T(\log_{10} T)\left[1 + \sum_{Si}^{2} k_{Si}\,(Gender)_S\right] + \sum_{Sj}^{2} k_{Sj}\,(Gender)_S \qquad (2)$$

Y_N is a normit,
Y_P is a probit,
k's are fitted coefficients,
C is vapor concentration, and
Both T and *Time* represent exposure-duration.

In eq 1, exposure-duration is treated as a three-level factor (*Time*), whereas in eq 2, exposure-duration is treated as a covariate (T). The constant $k_{T,S}$ has six values, one for each *Time-Gender* combination. The constants k_C and k_T are the probit slopes for concentration and time, respectively. The TL exponent, n, is the ratio k_C / k_T. If this ratio is not different (with statistical significance) from 1, then Haber's rule is appropriate for modeling the toxicity. Otherwise, the TL model (C^nT) is the proper approach, assuming that there is no significant curvature in the experimental data used to fit the model. Should significant curvature exist, the TL model is not appropriate, but it is still better than Haber's rule for modeling the data.

In addition to modeling the probability of a binary miotic response, change in pupil size through the use of a linear regression analysis of the individual fractional pupil sizes (a continuous response) was also modeled. The fractional pupil sizes were first transformed using a normit or Z transform, which allowed the models, developed for probability of miosis, to be applied to the fractional pupil size (see Section 3.5 for more details).

The present study has exposure-durations of 10, 60, and 180 min. For each exposure-duration, six to eight pigs of each gender were used. Statistical analysis routines, contained within Minitab® version 13 (Minitab, Inc., State College PA), and an in-house spreadsheet program were used for data analyses.

3. RESULTS

3.1 Animals.

A total of 38 pigs were exposed to concentrations of GF for miosis assessment of which 18 were male and 20 were female. At the time of the catheterization surgeries, the 18 males and 20 female pigs weighed an average of 10.43 kg ± 0.31 (SEM) kg and 10.66 ± 0.25 (SEM) kg, respectively.

3.2 Median Effective Dosages.

Pupil areas were calculated from images as described in Hulet *et al.*[9] At least five pre-exposure images were captured, and the average of the pupil areas of these images was used as the baseline pupil area. Table 1 shows the concentrations and durations of the exposures for both male and female pigs, the minimum pupil areas as a percent of their baseline values, the diameters from the minimum-area images as a percent of the baseline diameters, and the classification of each pig as having or not having miosis on an area basis and on a diameter basis. ECT_{50} values for miosis were estimated by maximum likelihood for male and female pigs at exposure-durations of 10, 60, and 180 min. The results (with approximate 95% confidence intervals) are found in Table 2 and shown in Figures 2-4.

The MLE analyses of the up and down method used an assumed probit slope of 10 to estimate the ECT_{50} separately for each gender exposure-duration group. No female pig exposed to GF for 180 min developed miosis on a diameter basis. One pig in the 180-min group (# 116), whose pupil diameter was 50.3% of baseline, was classified as having miosis in order to effect an MLE analysis by eq 1. For a particular duration, the ECT_{50} values on a diameter basis were greater than those on an area basis, with the exception of the 10-min ECT_{50} values in males, which were equal.

3.3 Time to Miosis.

The time to miosis begins at the start of the GF vapor exposure to the point the pupil area of a minipig becomes 50% or less of the pre-exposure pupil area. The values for TM were determined by plotting pupil area versus T for each minipig. Table 1 gives TM values for male and female pigs that developed miosis and the times to the minimum observed pupil area for each of the 38 pigs. The TM data for the 20 pigs (11 male and 9 female) exhibiting miosis on an area basis were analyzed by linear regression, with the logarithm of TM being regressed against the logarithm of the GF vapor concentration (C). In the regression model for time to miosis, the term for Sex was not statistically significant ($p = 0.082$). In the final fit (after dropping the term for Sex), it was found that TM equals $(5.678)(C)^{(-0.87)}$ with an R-squared of 78.9%. The 95% confidence interval for the exponent on C equals –0.67 to –1.07, which means that the dependence of TM on the value of C is not significantly different from a direct inverse proportion on C. It should be noted that the results for TM do not apply to the time to minimum observed pupil area. The logarithm of time to the minimum observed pupil area was a complicated function of the logarithms of the CT value of GF and the sex of the pig (see Appendix). All terms were statistically significant.

3.4 Statistical Models for the Probability of Miosis.

Several models were used to fit the quantal-response data shown in Table 1 in order to model the probability of miosis as a function of C, T, and gender (see Table 3 for model summary). The number of minipigs in each gender-exposure-duration group was not enough to estimate the response distribution for each group. Instead, the response distribution was estimated using either eq 1 or 2 with all of the data grouped together into one large data set for all 38 minipigs (see Section 2.10).

A total of six different combinations of model terms (the same for both area and diameter basis) were used, as listed in Table 3 and represented by eqs 1 and 2. Models A1, A2, A3, D1, D2 and D3 used T as a factor (Time), and as a continuous covariate (LogT) in A4, A5, A6, D4, D5 and D6. The fit for Model A5 is compared to MLE estimates for median effective dosages for miosis in males and females in Figure 3. A comparison is shown of the fits for Models A5 and D5 with the MLE estimates for median effective dosages for miosis in males and females in Figure 4.

Possible gender effects were tested for in Models A1, A2, A4, A5, D1, D2, D4 and D5. The gender term was found to be statistically significant, with the greater statistical significance for a diameter basis (a p-value of 0.003 for Model D2) than for an area basis (a p-value of 0.022 for Model A2). In practical terms, there is either a 32% difference (area basis-Model A5) or a 52% difference (diameter basis-Model D5) in the model fits for the miotic ECT_{50}'s between the genders. The males proved to be more sensitive than the females.

The time dependence of miosis (Haber's Rule, TL model or other) was tested for in Models A4, A5, A6, D4, D5 and D6. The value of the TL exponent ($n = k_C / k_T$) was dependent on the miosis basis used (either area or diameter). The values (range of 1.58 to 1.60) for the TL exponent for an area basis (Models A4, A5 and A6) were less than those (range of 1.77 to 1.87) for a diameter basis (Models D4, D5 and D6). Since these intervals do not overlap one, Haber's Law is not an appropriate time dependence model for this dataset. Potential curvature in the data was evaluated by inserting a $(LogT)^2$ term into Models A5 and D5 (see Appendix), and this term was found to be statistically insignificant (p-value > 0.3). Thus, the TL model is fully applicable to the dataset.

The steepness of the dose response curve (as represented by the probit slope (C), k_C) was initially assumed to equal 10 for the purposes of executing the up and down experimental design used in this study (see Section 2.10). The steepest probit slopes (C) were usually found for a particular set of terms in the model when T was treated as a factor rather than as a covariate (example for the terms LogC, T and Sex: 12.4 for Model A2 (T as a factor) versus 10.5 for Model A5 (T as a covariate)). This trend was also found in Hulet, *et al* (2006).[9] The probit slope (C) values range from 8.2 to 13.8 (area basis) and 5.5 to 18.5 (diameter basis) in the models with duration as a factor (see eq 1 and Table 3). When the standard errors for the probit slope values are considered, there is no statistically significant evidence from all six models (A1, A2, A3, D1, D2 and D3) to reject the original assumption that the probit slope equals 10. Thus, the binary normal regression results do not disprove the original assumption that the probit slope equals 10.

When eq 1 is fit to the quantal-response data of all 38 pigs for miosis on an area basis, the best model fit is provided by Models A2 and D2. The interaction of Sex*Log(T) was found to be statistically insignificant in Models A1 and D1. The lack of fit is greater in Models A3 and D3 due to the lack of a term (Sex) to explain gender differences. For Model A2 (area basis), the probit slope equals 12.4 with a standard error of 3.8; while for Model D2 (diameter basis), the probit slope is 14.6, with a standard error of 4.7.

Equation 2 was fit to the quantal-response data from both the area-basis classification (Model A5) of miosis and the diameter-basis classification (Model D5) of miosis. Because eq 2 does not use all degrees of freedom for the value of T, Sex, and interaction, it is not necessary to classify pig # 116 as having miosis on a diameter basis to fit eq 2 to the diameter-basis quantal-response data (as was done in Section 3.2). The model fits were (see Appendix for details):

$$Yn = 1.5076 + 0.6371\ Sex + 10.476\ LogC + 6.567\ LogT\ (Area\ Basis—A5) \quad (3)$$

$$Yn = 2.366 + 1.1713\ Sex + 12.973\ LogC + 7.312\ LogT\ (Diameter\ Basis—D5) \quad (4)$$

The TL exponents with approximate 95% confidence intervals were 1.60 (1.38, 1.82) for area-basis miosis and 1.77 (1.53, 2.01) for diameter-basis miosis. The higher-order interaction term, Sex*Log (T) (found in Models A4 and D4), was not statistically significant, thus Models A4 and D4 are not the best TL model fits for the data. Also, since the term, Sex, was significant in Models A5 and D5, Models A6 and D6 (which do not have a Sex term) are not adequate, either. So, Models A5 and D5 are the best TL model fits (see eqs 3 and 4 to the binary response data.

3.5 Statistical Models for Pupil Area and Pupil Diameter.

The measures of pupil size, the minimum observed pupil area divided by the baseline pupil area, and the length of the pupil's short axis at minimum pupil area divided by the baseline length of the short axis, normally vary between zero and one. Random variation in the measurements did not cause either pupil area or pupil diameter to exceed one. Therefore, the normit or probit transform, which is usually applied to the fraction of subjects showing a positive response, can be applied to the measures of pupil size. To distinguish applications of the normit transform to discrete data, such as the fraction of subjects with a positive response, from applications involving a continuous variable, such as fractional pupil size, the normit transform is referred to as the Z transform when it is applied to a continuous variable. The normit or Z transform is the inverse of the cumulative distribution function of the standard normal distribution. Any model of fractional pupil size as a function of CT, must account for the bounds on fractions. The Z transform allows the models developed for probability of miosis to be applied to fractional pupil size. The models for fractional pupil size are fit by linear regression because the dependent variables are continuous, whereas the models for probability of miosis (see Section 3.4) were fit by probit analysis or binary normal regression because the fraction of animals with miosis was discrete and had a binomial distribution. The linear regression model for pupil area, where sex is coded -1 for female and 1 for male, is

$$Z(area) = -0.901 - 0.365\ Sex - 4.47\ LogC - 2.70\ LogT \quad (5)$$

The residual standard deviation for eq 5 is 0.8060. The coefficients are negative because pupil area decreases with increasing CT values, whereas the probability of miosis increases with increasing CT values.

Several quantities can be derived from the model by noting the units of the model terms and the residual standard deviation. The dependent variable, Z(area), is in Z-units of area. The coefficient on LogC, 4.47, converts log-units of concentration (LUC) into Z-units of area (ZUA) and therefore must have units of ZUA/LUC. The residual standard deviation is the number of ZUA in one standard deviation (stdev) of the population; its units are ZUA/stdev. Dividing the coefficient on LogC by the residual standard deviation gives units of (ZUA/LUC)/(ZUA/stdev) = stdev/LUC. However, the number of standard deviations per one log unit of concentration is the probit slope. Therefore, the probit slope equals -4.466/0.8060, or -5.54. The ratio of the coefficient on LogC to the coefficient on LogT has units (ZUA/LUC)/(ZUA/LUT) = LUT/LUC, i.e. , the number of T log units (LUT) equivalent (in affecting the response) to one C log unit. This quantity is the TL exponent. Therefore, the TL exponent equals 4.47/2.70, or 1.66; an approximate 95% confidence interval for the TL exponent is (1.34, 1.97). Every term of eq 5 can be converted from Z units of pupil area to standard deviations of the population by dividing the coefficients by the residual standard deviation. Multiplying the converted coefficients by -1 will generate a model for the probability of miosis (rather than a model for the probability of not having miosis). The derived probit model is

$$Y_N = 1.11770 + 0.45234*\text{Sex} + 5.54036*\text{LogC} + 3.34554*\text{LogT} \qquad (6)$$

where the normit refers to the fraction of pigs with miosis, (i.e. the probability of miosis on an area basis). Equation 6 is conceptually the same as eq 3; the coefficients differ only because the two methods produce different estimates for the same quantities. In particular, the classification of pigs as having miosis or not, which was used for eq 3, appears to have reduced the error associated with measuring the pupil areas. Thus, the probit slopes are higher in eq 3 than in eq 6.

The process just described for modeling pupil area can be applied to pupil diameter. The resulting models are (the measurements of the short axis of the pupil are used):

$$Z(\text{diameter}) = -0.4485 - 0.3292*\text{Sex} - 4.205*\text{LogC} - 2.5160*\text{LogT} \qquad (7)$$

The residual standard deviation for eq 7 is 0.7460. The derived model for the probability of miosis on a diameter basis is

$$Y_N = 0.60121 + 0.44129*\text{Sex} + 5.63673*\text{LogC} + 3.37265*\text{LogT} \qquad (8)$$

where the probit slope of 5.63 has a standard error of 1.57 and the TL exponent of 1.67 (applicable to both eqs 7 and 8) has a standard error of 0.16 and an approximate 95% confidence interval (1.36, 1.99). The probit slopes and TL exponents estimated in eq 6 (denoted as Model ZA5) and eq 8 (denoted as Model ZD5) are summarized in Table 4.

3.6 GF Regeneration and Cholinesterase Inhibition.

Results of changes in cholinesterase activity and regenerated GF[12] in RBC and plasma fractions of the blood samples extracted throughout the nerve agent exposures will be included in a separate report.

4. DISCUSSION

4.1 IR Pupillometry.

Previous studies have propounded advantages of using IR pupillometry for quantifying pupil constriction in animals exposed to low-levels of nerve agent vapor.[6,9,18] During a vapor exposure, light is reflected off the subject's retina yielding the image of a bright pupil against a dark background (iris). Regardless of shape, IR pupillometry allows the calculation of pupil area, which is preferred over a simple measurement of pupil diameter since it is directly proportional to the quantity of light entering the eye.[19]

4.2 Median Effective Dosages.

A limited number of studies have investigated the effects of GF, particularly at concentrations low enough to result in only miosis. The results of the current study support the findings of Whalley et al. (2004)[6] who demonstrated that when rats are exposed to GF, CT (miosis) is not constant over time.[6] Evidence has been provided to support a similar scenario for the nerve agent GB.[8,20] The rats used in the GB study were as much as three times more sensitive to the nerve agent than the minipigs used in the present study.[8] However, only limited conclusions can be drawn between the various studies because two different techniques were used for quantifying pupil constriction.

4.3 Toxic Load Model Fit.

The best TL model fits for the current study (Models A5 and D5) produced TL (with corresponding 95% confidence ranges) of 1.60 (1.38 to 1.82) (area basis) and 1.77 (1.53 to 2.01) (diameter basis), respectively (Table 3). For whole body GB exposures in the minipig[9] a TL exponent value of 1.32 (with 95% confidence range of 1.14 to 1.50) was reported for both area and diameter basis models. On an area basis, the 95% confidence ranges between the GB and GF miosis exponent values overlap, indicating that there is no statistically significant difference between them. However, on a diameter basis, the 95% confidence ranges between the GB and GF exponent values do not overlap (though they come close). Thus, a statistically significant difference exists on a diameter basis. Nevertheless, for either case (area or diameter basis), the TL exponent value for GF miosis is greater than the TL exponent value for GB miosis. One possible explanation for this observation is that minipigs are refractory to GF for this endpoint (miosis) at the longer exposure-durations. In contrast, there was virtually no difference between the TL exponents for GF[6] and GB[8] induced pupil constriction in rats (1.98 and 1.96, respectively).

4.4 Gender Differences.

Female rats have been identified as being more sensitive than their male counterparts to lethal GF and GB vapor.[10, 21] A recent report identifying female rats as more sensitive than males to GF miosis levels[6] is consistent with GB miosis studies conducted on rats.[8] Female rats are consistently identified as being more sensitive than males to nerve agent effects. However, in the current study on minipigs, the males were significantly more sensitive to the pupil constricting effects of GF than the females. While the discovery that male pigs were more sensitive than female pigs is opposite of the nerve agent exposure in rats, it is not unique to the pigs. Male mice are consistently more sensitive to the effects of sarin vapor than females.[22,23] However, as both male and female pigs could not be housed simultaneously in the testing facility, there was no "true" randomization of the sexes during testing. This is one possible explanation for the statistical difference between the sexes at the longer T value.

Miosis elicited by vapor exposure to nerve agents is known to be caused by a direct local effect on the eye rather than a systemic effect.[1] The idea that one gender could be more susceptible to the effects of nerve agent on the pupil because of some gender-specific physiological difference is plausible. The most likely cause could arise from gender differences in the levels and/or activities of cholinesterase within the ocular tissues. However, no reports detailing gender related differences in cholinesterase activity in the eye for any species seem to exist. Gender differences in activities of other enzymes, such as sorbitol dehydrogenase and glucose-6-phosphate dehydrogenase,[24] and the structure of the tear-producing lacrimal gland[25] have been identified in a rat's eye. In humans, gender related differences have been identified in lacrimal gland structure,[26] intraocular pressure,[27] and Goblet Cell density.[28] The possibility that differences in intraocular pressure, tear-film production, or local enzyme acitivity may account for the gender differences in ECT_{50}'s identified in pigs in this study and with the rat in previous studies[6,8] needs to be investigated further.

4.5 GB/GF Potency Comparison.

Anthony *et al.* (2003)[10] described the relationship between the potency of GB and GF for lethal or highly toxic dosages of nerve agents in rats. In that study, GB and GF were approximately equipotent for the short duration of exposure (10-min) but as exposure-duration increased to 60 and 240 min, the potency of GF to GB increased to about 93 and 55%, respectively. No direct comparisons have yet been made between the potencies of GB and GF at "miosis-only" concentrations using the same species. The ECT_{50} values generated from GF vapor exposures in rats[6] could be compared to the ECT_{50} values generated from GB vapor exposures in rats.[8] However, the methods of data collection in the two studies were quite different so any conclusions drawn from comparing the data are tenuous at best. Table 5 shows EC_{50} values and potency comparisons for GB exposures in the minipig[9] compared to the values obtained for GF exposures in the current study. In male minipigs, GF is approximately equipotent for 60-min exposures and more potent for 10- and 180-min exposures. In the female minipig, GF is slightly more potent for the 10 min exposures but then progressively becomes less potent for the 60-and 180-min exposure-durations.

5. CONCLUSIONS

The present study utilized infrared pupillometry to digitally capture images of pupils in real-time during whole-body vapor GF exposures of Gottingen minipigs. Normal binary regression was used to fit various response models to the data. The EC_{50} and ECT_{50} values were calculated for miosis in male and female minipigs exposed to GF vapor for 10, 60 and 180 min. In practical terms, there is either a 32% overall difference (area basis) or a 52% overall difference (diameter basis) in the model fits for the miotic ECT_{50} values between the genders, with the males being more sensitive. The difference between the genders became more pronounced at the longer exposure-durations.

The ECT_{50} associated with miosis was not constant over time as predicted by Haber's rule. Rather, the data were best described by a toxic load model. The value of the best area basis model fit for the toxic load exponent was 1.60 with a 95% confidence interval of 1.38 to 1.82. The best estimate of the probit slope (C) was 12.4 with 95% confidence interval of 4.8 to 20.0. Potential curvature in the data with respect to fitting by the toxic load model was evaluated by inserting the term, $(LogT)^2$, and this term was found to be statistically insignificant ($p > 0.3$).

The time to miosis (TM) was found to equal $(5.678)(C)^{(-0.87)}$ with an R-square of 78.9% for the linear regression fit. The 95% confidence interval for the exponent on C equals –0.67 to – 1.07, which means that the dependence of TM on vapor concentration is not significantly different from a direct inverse proportion to the value of C.

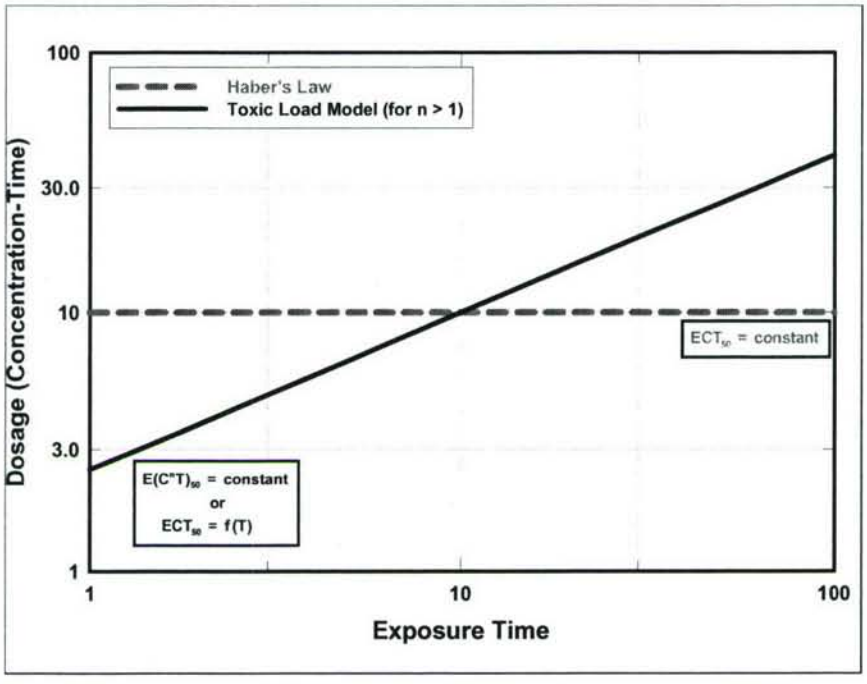

Figure 1. Comparison of Haber's Law and Toxic Load Models for Toxicity Time Dependence

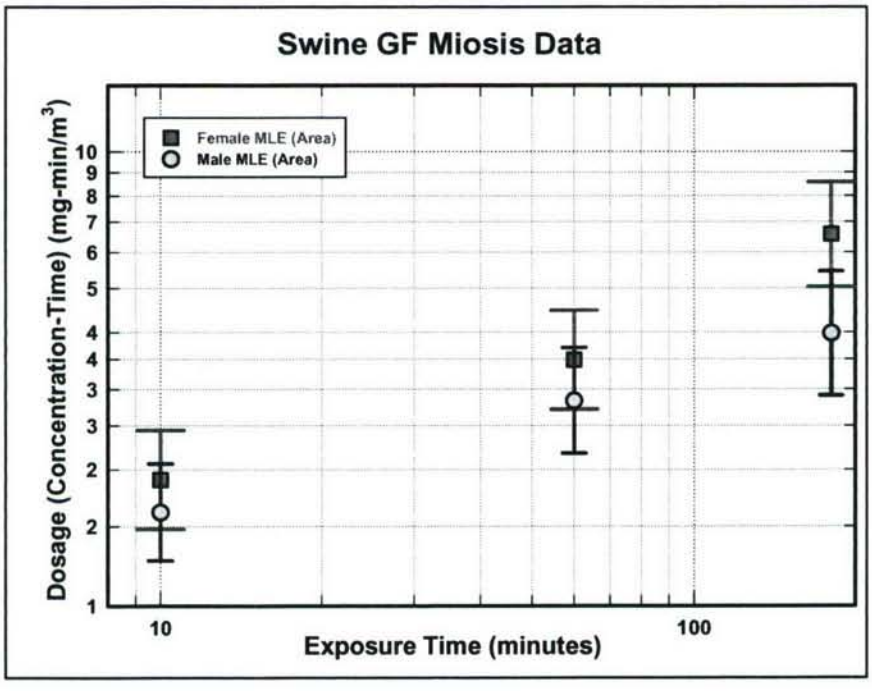

Figure 2. MLE ECT_{50} Miosis Estimates (Area Basis) for Male and Female Minipigs as a Function of Exposure-Duration with Accompanying Asymptotic 95% Confidence Intervals

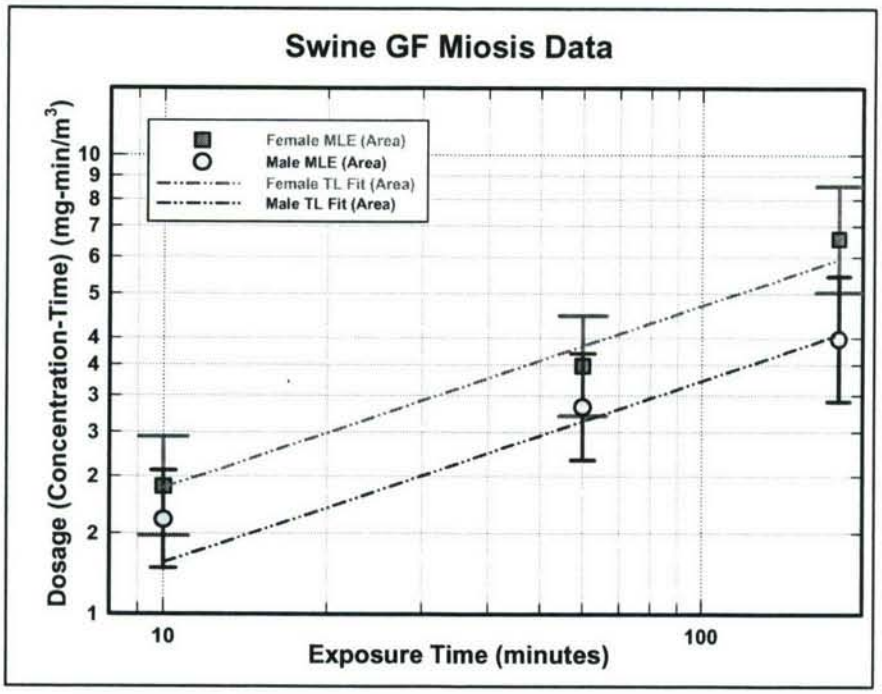

Figure 3. Comparison of Model A5 (Classic Toxic Load) Fit and MLE ECT_{50} Miosis Estimates (Area Basis) for Male and Female Minipigs

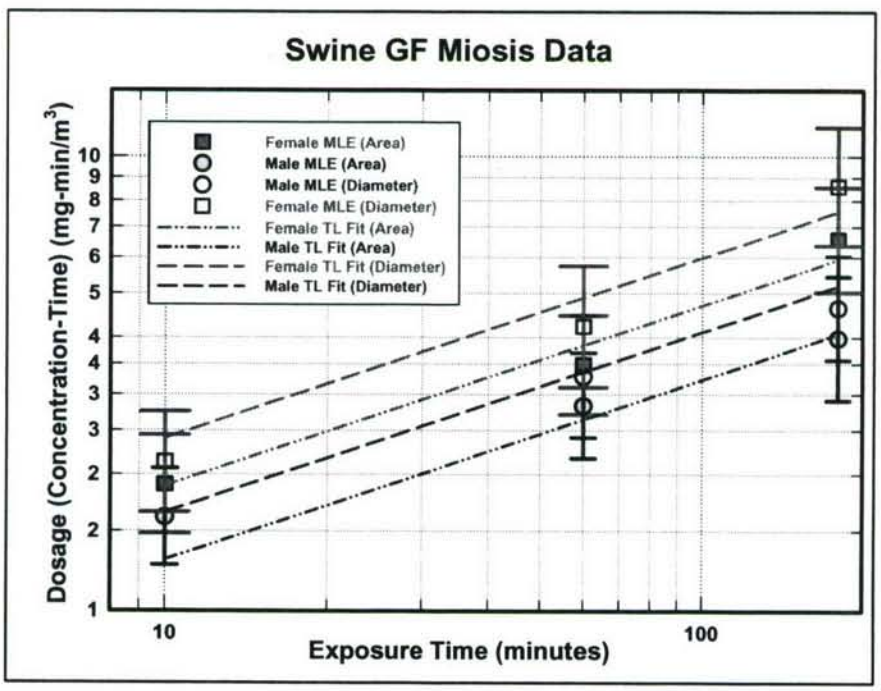

Figure 4. Comparison of Models A5 and D5 (Classic Toxic Load) Fit and MLE ECT_{50} Miosis Estimates (for Both Area and Diameter Bases) for Male and Female Minipigs

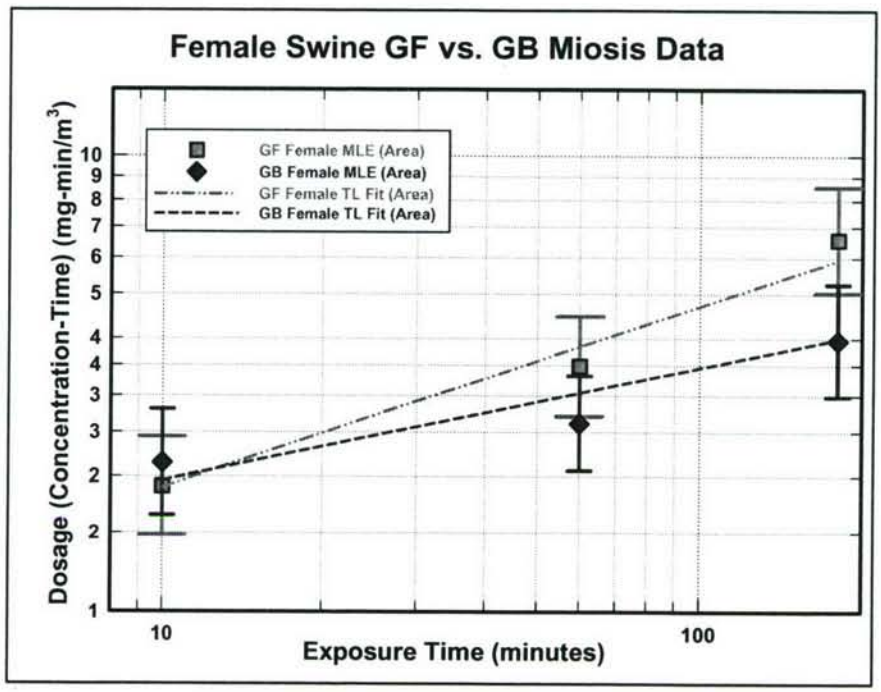

Figure 5. Comparison of Miosis Estimates (Area Basis) for Exposure of Female Minipigs to Vapor GB and GF

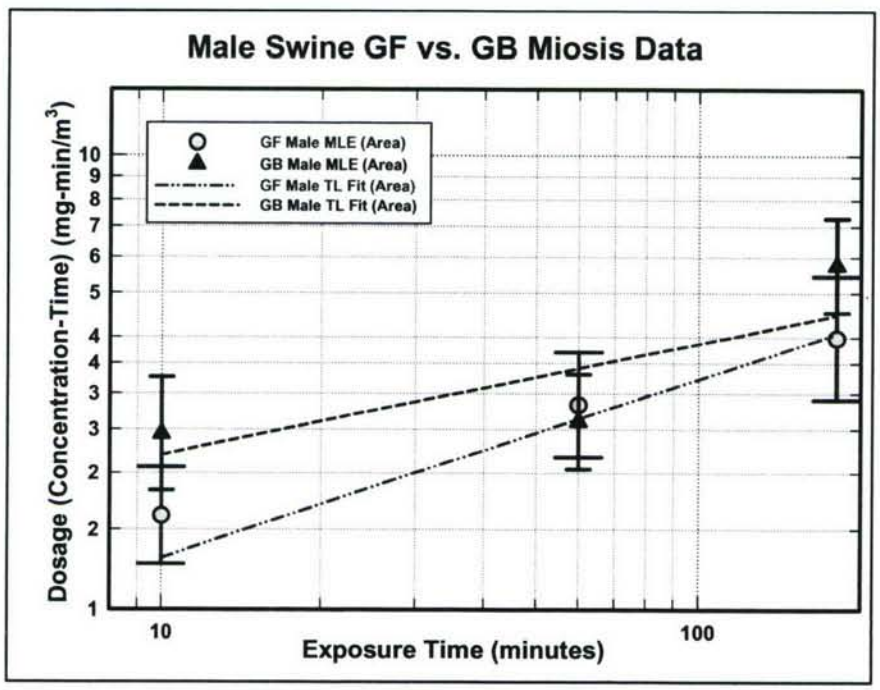

Figure 6. Comparison of Miosis Estimates (Area Basis) for Exposures of Male Minipigs to Vapor GB and GF

Table 1. Durations and Concentrations of GF Exposures for Male (18) and Female (20) Minipigs and the Incidence of Miosis (O = no miosis, X = miosis)

Pig #	Sex	GF Conc. (mg/m^3)	Time (min)	CT ($mg.min/m^3$)	Miosis (1=yes, 0=no)	Time to Miosis (hh:mm:ss)	Max Miosis (% baseline)	Time to Max (hh:mm:ss)
109	f	0.190	10	1.90	0	X	92	0:26:52
108	f	0.240	10	2.40	1	0:25:29	26	0:29:41
102	f	0.180	10	1.80	1	0:18:26	11	0:23:25
118	f	0.180	10	1.80	0	X	68	0:19:16
120	f	0.210	10	2.10	1	0:30:52	39	0:35:47
117	f	0.150	10	1.50	0	X	89	0:16:30
105	f	0.036	60	2.16	0	X	68	1:16:18
101	f	0.042	60	2.52	1	1:14:49	40	1:14:55
107	f	0.037	60	2.22	0	X	62	1:38:29
106	f	0.045	60	2.70	0	X	62	1:11:16
112	f	0.060	60	3.60	0	X	84	1:20:22
111	f	0.081	60	4.86	1	0:41:38	16	1:46:52
115	f	0.061	60	3.66	0	X	74	1:10:57
119	f	0.079	60	4.74	1	0:36:01	6	0:51:37
103	f	0.027	180	4.86	0	X	77	3:18:09
110	f	0.034	180	6.12	0	X	66	3:42:02
104	f	0.043	180	7.74	1	2:18:40	43	3:03:35
114	f	0.041	180	7.38	1	2:54:12	39	3:48:32
113	f	0.034	180	6.12	0	X	77	3:10:11
116	f	0.041	180	7.38	1	3:04:16	34	3:12:34
100	m	0.180	10	1.80	0	X	78	0:52:01
91	m	0.220	10	2.20	1	0:20:51	9	0:55:19
99	m	0.210	10	2.10	1	0:22:51	22	0:47:22
129	m	0.180	10	1.80	1	0:20:37	5	0:28:50
125	m	0.160	10	1.60	1	0:22:12	7	0:57:34
127	m	0.140	10	1.40	0	X	61	0:52:17
95	m	0.035	60	2.10	0	X	55	1:17:44
94	m	0.040	60	2.40	0	X	92	0:57:50
96	m	0.048	60	2.88	1	1:12:43	45	1:15:48
124	m	0.040	60	2.40	0	X	75	1:28:39
123	m	0.050	60	3.00	1	1:04:20	13	1:12:13
121	m	0.040	60	2.40	0	X	87	1:52:00
92	m	0.028	180	5.04	1	1:52:56	47	2:17:38
98	m	0.033	180	5.94	1	2:14:01	13	3:05:14
122	m	0.024	180	4.32	0	X	79	3:15:58
128	m	0.032	180	5.76	1	2:19:32	26	3:18:24
126	m	0.025	180	4.50	1	1:06:49	5	3:11:47
130	m	0.035	180	6.30	1	1:11:00	8	3:11:37

Table 2. Maximum Likelihood Estimates for Median Effective Concentrations and Dosages with Approx. 95% Confidence Intervals for GF 10-, 60-, and 180-min Exposure-Durations

Area Basis

	Males			Females		
Exposure-Duration (min)	EC_{50}	ECT_{50}	95% Limits	EC_{50}	ECT_{50}	95% Limits
10	0.161	1.61	1.26-2.06	0.190	1.90	1.48-2.44
60	0.047	2.83	2.17-3.70	0.058	3.48	2.71-4.47
180	0.022	3.98	2.91-5.45	0.037	6.57	5.03-8.56

Diameter Basis

	Males			Females		
Exposure-Duration (min)	EC_{50}	ECT_{50}	95% Limits	EC_{50}	ECT_{50}	95% Limits
10	0.161	1.61	1.26-2.06	0.213	2.13	1.65-2.75
60	0.055	3.28	2.41-4.47	0.071	4.23	3.11-5.74
180	0.026	4.64	3.57-6.04	0.048	8.60	6.37-11.61

Table 3. Probit Slopes and Toxic Load Exponents (n) Obtained from Various Binary Normal Regression Model Fits for Miosis Probability

ID	Basis	Terms in Model	k_C	SE(C)	k_T	SE(T)	n	SE(n)
A1	Area	LogC Time Sex Time*Sex	13.8	4.4	---	---	---	---
A2	Area	LogC Time Sex	12.4	3.8	---	---	---	---
A3	Area	LogC Time	8.2	2.8	---	---	---	---
A4	Area	LogC LogT Sex LogT*Sex	11.2	3.4	7.1	2.2	1.58	0.11
A5	Area	LogC LogT Sex	10.5	3.1	6.6	2.0	1.60	0.11
A6	Area	LogC LogT	7.9	2.4	5.0	1.6	1.58	0.14
D1	Diameter	LogC Time Sex Time*Sex	18.5	6.0	---	---	---	---
D2	Diameter	LogC Time Sex	14.6	4.7	---	---	---	---
D3	Diameter	LogC Time	5.5	2.6	---	---	---	---
D4	Diameter	LogC LogT Sex LogT*Sex	17.2	5.8	9.7	3.4	1.77	0.10
D5	Diameter	LogC LogT Sex	13.0	4.3	7.3	2.5	1.77	0.12
D6	Diameter	LogC LogT	5.6	2.3	3.0	1.5	1.87	0.28

Table 4. Probit Slopes and Toxic Load Exponents (n) Obtained from Linear Regression Model Fits Using Z Transform of Fractional Pupil Size Measurements

ID	Basis	Terms in Model	k_C	SE(C)	k_T	SE(T)	n	SE(n)
ZA5	Area	LogC LogT Sex	5.5	1.6	3.3	1.0	1.66	0.16
ZD5	Diameter	LogC LogT Sex	5.6	1.6	3.4	1.0	1.67	0.16

Table 5. Relative ECT_{50} (Miosis) Potencies for GB vs. GF Whole-Body Vapor Exposures in Male and Female Gottingen Minipigs

	Males			Females		
Duration (min)	GF ($mg.min/m^3$)	GB ($mg.min/m^3$)	GF/GB Ratio	GF ($mg.min/m^3$)	GB ($mg.min/m^3$)	GF/GB Ratio
10	1.61	2.44	0.66	1.90	2.14	0.89
60	2.83	2.60	1.09	3.48	2.61	1.33
180	3.98	5.75	0.69	6.57	3.96	1.66

LITERATURE CITED

1. Sim, V.M. Effect on Pupil Size of Exposure to GB Vapour. *CDE PTP* **1956**, *531*.

2. Kato, T.; Hamanaka, T. Ocular Signs and Symptoms Caused by Exposure to Sarin Gas. *Am. J. Opthalmol.* **1996**, *121*, p 209.

3. Salem, H. Principles of Inhalation Toxicology. In *Inhalation Toxicology: Research Methods, Applications, and Evaluation,* Salem, H., Ed.; Marcel Dekker, Inc.: New York, 1987; pp 1-33.

4. Haber, F.R. Zur Geschichte des Gaskrieges. In *Funf Vortrage aus Jahren*, 1920-1923; Spinger, Berlin: 1924.

5. ten Berge, W.F.; Zwart, A.; Appelman, L.M. Concentration-Time Mortality Response Relationship of Irritant and Systemically Acting Vapours and Gases. *Journal of Hazardous Materials* **1986**, *13*, pp 301-309.

6. Whalley, C.E.; Benton, B.J.; Manthei, J.H.; Way, R.A.; Jakubowski, E.M., Jr.; Burnett, D.C.; Gaviola, B.I.; Crosier, R.B.; Sommerville, D.R.; Muse, W.T.; Forster, J.S.; Mioduszewski, R.J.; Thomson, S.A.; Scotto, J.A.; Miller, D.B.; Crouse, C.L.; Matson, K.L.; Edwards, J.L. *Low-Level Cycolosarin (GF) Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size*; ECBC-TR-407; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2004; UNCLASSIFIED Report (AD A429 234).

7. Sommerville, D.R.; Park, K.H.; Kierzewski, M.O.; Dunkel, M.D.; Hutton, M.I.; Pinto, N.A. Toxic Load Modeling. In *Inhalation Toxicology*, 2nd ed.; Salem, H., Ed.; Marcel Dekker: New York, submitted for publication, 2005.

8. Mioduszewski, R.J.; Manthei, J.H.; Way, R.A.; Burnett, D.C.; Gaviola, B.I.; Muse, W.T.; Thomson, S.A.; Sommerville, D.R.; Crosier, R.B.; Scotto, J.A.; McCaskey, D.A.; Crouse, C.L.; Matson, K.L. *Low-Level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size*; ECBC-TR-235; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2002; UNCLASSIFIED Report (AD A402 869).

9. Hulet, S.W.; Sommerville, D.R.; Benton, B.J.; Forster, J.S.; Scotto, J.A.; Way, R.A.; Muse, W.T.; Crosier, R.B.; Reutter, S.A.; Miller, D.B.; Jarvis, J.R.; Mioduszewski, R.J.; Thomson, S.A. *Low-Level Sarin (GB) Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size*; ECBC-TR-450; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2006; UNCLASSIFIED Report (AD A458 520).

10. Anthony, J.S.; Haley, M.V.; Manthei, J.H.; Way, R.A.; Burnett, D.C.; Gaviola, B.P.; Sommerville, D.R.; Crosier, R.B.; Mioduszewski, R.J.; Thomson, S.A.; Crouse, C.L.; Matson, K.L. *Inhalation Toxicity of GF Vapor in Rats as a Function of Exposure Concentration and Duration and Its Potency Comparison to GB*; ECBC-TR-335; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2003; UNCLASSIFIED Report (AD A417 763).

11. *Information Resources on Swine in Biomedical Science*; Smith, C.P., Ed.; United State Department of Agriculture: Beltsville, MD, 2000.

12. Jakubowski, E.M.; Heykamp, L.S.; Mioduszewski, R.J.; Manthei, J.H.; Way, R.A.; Burnett, D.C.; Gaviola, B.I.; Muse, W.T.; Anthony, J.; Crouse, C.; Durst, H.D.; Thomson, S.A. *Analysis of Rat Blood Samples for Agent Biomarkers After GB Inhalation Exposure*, Proceedings of the 2001 Chemical Biological Defense Conference, Hunt Valley, MD, March 8, 2001.

13. Miller, D.B.; Benton, B.J.; Hulet, S.W.; Mioduszewski, R.J.; Whalley, C.E.; Carpin, J.C.; Thomson, S.A. *An Infrared Image Acquisition and Analysis Method for Quantifying Optical Response to Chemical Agent Vapor Exposure.* Proceedings of the 2002 Joint Service Scientific Conference on Chemical & Biological Defense Research. Hunt Valley, MD, November 19-21, 2002.

14. Bruce, R.D. An Up-and-Down Procedure for Acute Toxicity Testing. *Fundam. Appl. Toxicol.* **1985**, *5*, pp 151-157.

15. Fox, J. *Applied Regression Analysis, Linear Models, and Related Methods;* Sage Publications, Inc.: Thousand Oaks, CA, 1997.

16. Finney, D.J. *Probit Analysis*; 3rd ed.; Cambridge University Press: Cambridge, 1971.

17. Agresti, A. *Categorical Data Analysis*; John Wiley & Sons: New York, 1990.

18. Benton, B.J.; Sommerville, D.R.; Scotto, J.; Jarvis, J.; Burnett, D.C.; Gaviola, B.I.; Crosier, R.B.; Jakubowski, E.M., Jr.; Whalley, C.E.; Anthony, J.S., Jr.; Hulet, S.W.; Dabisch, P.A.; Reutter, S.A.; Forster, J.S.; Mioduszewski, R.J.; Thomson, S.A.; Matson, K.L.; Crouse, C.L.; Miller, D.; Evans, R.A.; McGuire, J.M. *Low-Level Effects of VX Vapor Exposure on Pupil Size and Cholinesterase Levels in Rats*; ECBC-TR-428; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2005; UNCLASSIFIED Report (AD A432 945).

19. Murray, R.B.; Loughnane, M.H. Infrared Video Pupillometry: A Method Used to Measure the Papillary Effects of Drugs in Small Laboratory Animals in Real Time. *J. Neuroscience Methods* **1981**, *3*, pp 365-375.

20. McKee, W.H.E.; Woolcott, B. Report on Exposures of Unprotected Men and Rabbits to Low Concentrations of Nerve Gas Vapour. *PRP-143*, Porton Down, 1949.

21. Mioduszewski, R.; Manthei, J.; Way, R.; Burnett, D.; Gaviola, B.; Muse, W.; Thomson, S.; Sommerville, D.; Crosier, R. Interaction of Exposure Concentration and Duration in Determining Acute Toxic Effects of Sarin Vapor in Rats. *Toxicol. Sci.* **2002**, *66*, pp 176-184.

22. Long, E.K.; Thompson O.A.; Woolsey R.D. Dispersion of GB from Miniature Bombs in an Explosion Chamber. *Chem Corps Chem and Rad Lab* **1951**.

23. McPhail, M.K. *Sex and the Response to G Agents*; Suffield Technical Report No. 38, 1953.

24. Bours J.; Fink H.; Hockwin O. The Quantification of Eight Enzymes from the Ageing Rat Lens with Respect to Sex Differences and Special Reference to Aldolase. *Curr. Eye. Res.* **1988**, *7(5)*, pp 449-55.

25. Sullivan, D.A.; Hann L.E.; Yee L.; Allansmith, M.R. Age and Gender-Related Influence on the Lacrimal Gland and Tears. *Acta Opthalmol (Copenh)* **1990,** *68(2)*, pp 188-194.

26. Ueno, H.; Ariji, E.; Izumi, M.; Uetani, M.; Havashi, K.; Nakamura, T. MR Imaging of the Lacrimal Gland. Age Related and Gender Dependent Changes in Size and Structure. *Acta Radiol.* **1996**, *37(5)*, pp 714-9.

27. Qureshi, I.A. Intraocular Pressure: A Comparative Analysis in Two Sexes. *Clin Physiol.* **1997**, *17(3)*, pp 247-55.

28. Connor C.G.; Flockencier L.L.; Hall C.W. The Influence of Gender on the Ocular Surface. *J Am Optom Assoc.* **1999**, *70(3)*, pp 182-6.

Blank

APPENDIX
PRINTOUTS FROM MINITAB®

1.0 INTRODUCTION

Three statistical analysis routines in MINITAB® were used on the total dataset (both genders and all three exposure-durations): traditional probit analysis (Section 2.0), binary logistic regression with a normit link function (Section 3.0), and linear regression (Section 4.0). In the present study, all three methods produced approximately the same estimates for median effective dosages for the six gender-time groups. The binary logistic regression routine in MINITAB® is able to estimate the coefficients needed to calculate the toxic load exponent, whereas the probit analysis routine cannot estimate the required coefficients. Linear regression was used to model pupil size as well as the time to miosis and the time to minimum observed pupil area.

NOMENCLATURE

IDIdentification number of test animal
Gender/SexM or 1 for Male and F or -1 for Female
CGF vapor concentration (mg/m^3)
TExposure-duration (min)
CTConcentration-time ($mg\text{-}min/m^3$)
logCTLogarithm (Base 10) of CT
logCLogarithm (Base 10) of C
LogTLogarithm (Base 10) of T
ECT50Effective Concentration-time to cause effect in 50% of exposed pigs
PercentAPercent of pre-exposure pupil area (minimum observed)
PercentD Percent of pre-exposure pupil diameter (based on short axis of pig's eye)
MiosisA Miosis indicator for an exposed pig based upon pupil area constriction:
0 for pupil area constriction less than 50%
1 for pupil area constriction equal to or greater than 50%
MiosisDMiosis indicator for an exposed pig based upon pupil diameter constriction:
0 for pupil diameter constriction less than 50%
1 for pupil diameter constriction equal to or greater than 50%
Group:Gender exposure-duration combinations:
F10:Female--10-min exposure-duration
M10:Male--10- min exposure-duration
F60:Female--60-min exposure-duration
M60:Male--60-min exposure-duration
F180:Female--180-min exposure-duration
M180:Male--180-min exposure-duration

Data Display – Table 1

Row	ID	Group	Conc	CT	PercentA	MiosisA	PercentD	MiosisD
1	109	F10	0.190	1.90	92	0	95.4	0
2	108	F10	0.240	2.40	26	1	47.5	1
3	102	F10	0.180	1.80	11	1	26.5	1
4	118	F10	0.180	1.80	68	0	81.5	0
5	120	F10	0.210	2.10	39	1	55.2	0
6	117	F10	0.150	1.50	89	0	91.4	0
7	105	F60	0.036	2.16	68	0	82.5	0
8	101	F60	0.042	2.52	40	1	59.6	0
9	107	F60	0.037	2.22	62	0	72.4	0
10	106	F60	0.045	2.70	62	0	75.0	0
11	112	F60	0.060	3.60	84	0	91.5	0
12	111	F60	0.081	4.86	16	1	31.1	1
13	115	F60	0.061	3.66	74	0	77.6	0
14	119	F60	0.079	4.74	6	1	17.4	1
15	103	F180	0.027	4.86	77	0	96.1	0
16	110	F180	0.034	6.12	66	0	77.4	0
17	104	F180	0.043	7.74	43	1	54.3	0
18	114	F180	0.041	7.38	39	1	54.7	0
19	113	F180	0.034	6.12	77	0	84.1	0
20	116	F180	0.041	7.38	34	1	50.3	0*
21	100	M10	0.180	1.80	78	0	88.2	0
22	91	M10	0.220	2.20	9	1	20.7	1
23	99	M10	0.210	2.10	22	1	36.8	1
24	129	M10	0.180	1.80	5	1	16.2	1
25	125	M10	0.160	1.60	7	1	17.9	1
26	127	M10	0.140	1.40	61	0	75.1	0
27	95	M60	0.035	2.10	55	0	68.4	0
28	94	M60	0.040	2.40	92	0	97.6	0
29	96	M60	0.048	2.88	45	1	58.2	0
30	124	M60	0.040	2.40	75	0	83.7	0
31	123	M60	0.050	3.00	13	1	27.5	1
32	121	M60	0.040	2.40	87	0	92.5	0
33	92	M180	0.028	5.04	47	1	70.2	0
34	98	M180	0.033	5.94	13	1	28.0	1
35	122	M180	0.024	4.32	79	0	87.5	0
36	128	M180	0.032	5.76	26	1	43.8	1
37	126	M180	0.025	4.50	5	1	15.1	1
38	130	M180	0.035	6.30	8	1	19.5	1

* 1 was substituted for 0 when necessary to obtain an analysis; see text.

2.0 PROBIT ANALYSIS OF MIOSIS VERSUS DOSAGE AND GROUP

2.1 Miosis on Area Basis as a Function of CT and Group

Probit Analysis: MiosisA versus CT, Group

```
Distribution:  Lognormal base 10

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Factor Information

Factor   Levels Values
Group         6 F10  F180 F60  M10  M180 M60

Estimation Method:  Maximum Likelihood

Regression Table
                      Standard
Variable        Coef      Error        Z      P
Constant      -3.811      1.303    -2.93 0.003
CT            13.768      4.223     3.26 0.001
Group
 F180         -7.467      2.482    -3.01 0.003
 F60          -3.517      1.275    -2.76 0.006
 M10          0.8969     0.8643     1.04 0.299
 M180         -4.748      1.931    -2.46 0.014
 M60          -2.265      1.040    -2.18 0.029
Natural
Response       0.000

Test for equal slopes:  Chi-Square = 8.2104,  DF = 5,  P-Value = 0.145
Log-Likelihood = -15.544

Multiple degree of freedom test

Term      Chi-Square    DF      P
Group         11.503     5  0.042

Goodness-of-Fit Tests

Method                Chi-Square    DF      P
Pearson                   40.390    25  0.027
Deviance                  25.543    25  0.432
```

Listing of Median Effective Dosages (denoted "Percentile" below)

Female Swine, 10-min exposure-duration

```
                      Standard       95.0% Fiducial CI
 Percent  Percentile       Error       Lower       Upper
      50      1.8916      0.1861      1.4892      2.4127
```

Female Swine, 180-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	6.5944	0.6956	5.0426	8.4629

Female Swine, 60-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	3.4064	0.3459	2.7502	4.5517

Male Swine, 10-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	1.6281	0.1692	1.2184	2.0372

Male Swine, 180-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	4.1846	0.5409	2.8269	5.4067

Male Swine, 60-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	2.7627	0.2951	2.1988	3.7313

Potency Comparison between the Six Levels of Group

Note: If the 95% fiducial CI does not overlap 1.0, then there is a statistically significant difference between the two group levels being compared.

Table of Relative Potency

Factor: Group

Comparison	Relative Potency	95.0% Fiducial CI Lower	Upper	
F10 VS F180	3.4861	2.4189	4.9103	
F10 VS F60	1.8008	1.3178	2.6439	
F10 VS M10	0.8607	0.5839	1.1831	not different
F10 VS M180	2.2122	1.3755	3.0926	
F10 VS M60	1.4605	1.0571	2.1602	
F180 VS F60	0.5166	0.3764	0.7794	
F180 VS M10	0.2469	0.1678	0.3465	
F180 VS M180	0.6346	0.3969	0.9023	different
F180 VS M60	0.4189	0.3021	0.6364	
F60 VS M10	0.4780	0.3078	0.6442	
F60 VS M180	1.2284	0.7205	1.6946	
F60 VS M60	0.8110	0.5678	1.1542	not different
M10 VS M180	2.5702	1.6658	3.6965	
M10 VS M60	1.6969	1.2463	2.6521	
M180 VS M60	0.6602	0.4745	1.1312	

2.2 Miosis on Diameter Basis as a Function of CT and Group

Probit Analysis: MiosisD versus CT, Group

Note: pig 116 classified as having miosis for this analysis

```
Distribution:  Lognormal base 10

Response Information

Variable  Value       Count
MiosisD   1             14  (Event)  Note: Count = 14 instead of 13 due to pig 116.
          0             24
          Total         38

Factor Information

Factor   Levels Values
Group         6 F10  F180 F60  M10  M180 M60

Estimation Method:  Maximum Likelihood

Regression Table
                        Standard
Variable        Coef       Error        Z      P
Constant      -5.735       1.916    -2.99 0.003
CT            18.509       6.293     2.94 0.003
Group
 F180        -10.931       3.754    -2.91 0.004
 F60          -5.750       2.339    -2.46 0.014
 M10          1.7057      0.9558     1.78 0.074
 M180         -6.864       2.677    -2.56 0.010
 M60          -3.131       1.390    -2.25 0.024
Natural
Response       0.000

Test for equal slopes:  Chi-Square = 1.7404,  DF = 5,  P-Value = 0.884
Log-Likelihood = -12.729

Multiple degree of freedom test

Term      Chi-Square    DF      P
Group         10.364     5  0.066

Goodness-of-Fit Tests

Method                Chi-Square    DF      P
Pearson                   14.247    25  0.957
Deviance                  17.140    25  0.877
```

Listing of Median Effective Dosages (denoted "Percentile" below)

Female Swine, 10-min exposure-duration

```
                        Standard       95.0% Fiducial CI
 Percent   Percentile       Error       Lower       Upper
      50       2.0411      0.1590      1.7234      2.6150
```

Female Swine, 180-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	7.9509	0.7289	6.5994	10.8595

Female Swine, 60-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	4.1737	0.4485	3.1502	5.5425

Male Swine, 10-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	1.6508	0.1348	1.2905	1.9907

Male Swine, 180-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	4.7939	0.3902	3.8059	5.8458

Male Swine, 60-min exposure-duration

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	3.0132	0.3031	2.4491	4.2223

Potency Comparison between the Six Levels of Group

Note: If the 95% fiducial CI does not overlap 1.0, then there is a statistically significant difference between the two group levels being compared.

Table of Relative Potency

Factor: Group

Comparison	Relative Potency	95.0% Fiducial CI Lower	Upper	
F10 VS F180	3.8954	2.9330	5.4219	
F10 VS F60	2.0449	1.3860	2.7954	
F10 VS M10	0.8088	0.5541	1.0287	not different
F10 VS M180	2.3487	1.6396	3.0111	
F10 VS M60	1.4763	1.0937	2.0981	
F180 VS F60	0.5249	0.3388	0.7190	
F180 VS M10	0.2076	0.1344	0.2667	
F180 VS M180	0.6029	0.3983	0.7794	different
F180 VS M60	0.3790	0.2695	0.5354	
F60 VS M10	0.3955	0.2686	0.5478	
F60 VS M180	1.1486	0.7916	1.6097	
F60 VS M60	0.7219	0.5200	1.1389	not different
M10 VS M180	2.9039	2.1770	3.9782	
M10 VS M60	1.8252	1.3994	2.8766	
M180 VS M60	0.6285	0.4788	0.9708	

3.0 BINARY LOGISTIC REGRESSION ANALYSIS OF MIOSIS VERSUS EXPOSURE CONCENTRAION, EXPOSURE-DURATION, AND GENDER

These analyses were used to calculate a toxic load exponent for miosis. Error estimates for the probit slope and toxic load exponent were also obtained. A summary of the probit slope values and error estimates is provided in Table 4.

3.1 Miosis on Area Basis

Model A1: Binary Logistic Regression of MiosisA versus LogC, T, Sex, T*Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Constant      10.405      3.270      3.18  0.001
LogC          13.768      4.361      3.16  0.002
T
  60           7.374      2.529      2.92  0.004
 180          10.727      3.371      3.18  0.001
Sex           0.4485     0.4294      1.04  0.296
T*Sex
  60          0.1777     0.6466      0.27  0.783
 180          0.9113     0.6702      1.36  0.174

Log-Likelihood = -15.544
Test that all slopes are zero: G = 21.485, DF = 6, P-Value = 0.002

Goodness-of-Fit Tests

Method                Chi-Square    DF       P
Pearson                   40.390    25   0.027
Deviance                  25.543    25   0.432
Hosmer-Lemeshow            6.289     8   0.615
```

Model A2: Binary Logistic Regression of Miosis A versus LogC, T, Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
```

```
Constant        9.393       2.856      3.29 0.001
LogC           12.384       3.784      3.27 0.001
T
  60            6.567       2.198      2.99 0.003
 180            9.609       2.920      3.29 0.001
Sex            0.7198      0.3131      2.30 0.022

Log-Likelihood = -16.592
Test that all slopes are zero: G = 19.390, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
Pearson                    42.204    27  0.031
Deviance                   27.638    27  0.430
Hosmer-Lemeshow            24.835     8  0.002
```

Model A3: Binary Logistic Regression of MiosisA versus LogC, T

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Constant       6.284      2.127      2.95 0.003
LogC           8.224      2.831      2.90 0.004
T
  60           4.143      1.726      2.40 0.016
 180           6.476      2.239      2.89 0.004

Log-Likelihood = -19.991
Test that all slopes are zero: G = 12.591, DF = 3, P-Value = 0.006

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
Pearson                    29.776    26  0.277
Deviance                   34.438    26  0.124
Hosmer-Lemeshow            17.288     8  0.027
```

Model A4: Binary Logistic Regression of MiosisA versus LogC, LogT, Sex, LogT*Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table
```

```
Predictor       Coef    SE Coef        Z      P
Constant      1.5459     0.9385     1.65  0.099
LogC          11.235      3.397     3.31  0.001
LogT           7.086      2.165     3.27  0.001
Sex           -0.2492     0.8170    -0.30  0.760
LogT*Sex       0.5607     0.5022     1.12  0.264

Log-Likelihood = -16.417
Test that all slopes are zero: G = 19.740, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method                Chi-Square    DF      P
Pearson                   64.275    27  0.000
Deviance                  27.288    27  0.448
Hosmer-Lemeshow           42.600     8  0.000

 Variance-Covariance Matrix of the Estimated Parameters

  Constant    LogC      LogT       Sex    LogT*Sex
    0.8807  1.5902    0.6209    0.0176    0.0416 Constant
    1.5902 11.5406    7.1763   -0.2612    0.5586 LogC
    0.6209  7.1763    4.6877   -0.1978    0.3715 LogT
    0.0176 -0.2612   -0.1978    0.6675   -0.3805 Sex
    0.0416  0.5586    0.3715   -0.3805    0.2522 LogT*Sex
```

Model A5: Binary Logistic Regression of MiosisA versus Sex, LogC, LogT

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Constant      1.5076     0.9461     1.59  0.111
Sex           0.6371     0.2969     2.15  0.032
LogC          10.476      3.134     3.34  0.001
LogT           6.567      1.978     3.32  0.001

Log-Likelihood = -17.055
Test that all slopes are zero: G = 18.464, DF = 3, P-Value = 0.000

Goodness-of-Fit Tests

Method                Chi-Square    DF      P
Pearson                   47.224    28  0.013
Deviance                  28.564    28  0.435
Hosmer-Lemeshow           27.362     8  0.001

Variance-Covariance Matrix of the Estimated Parameters

  0.89509   0.09193   1.50046   0.55531
  0.09193   0.08813   0.53422   0.32234
  1.50046   0.53422   9.81960   6.02073
  0.55531   0.32234   6.02073   3.91065
```

Model A6: Binary Logistic Regression of MiosisA versus LogC, LogT

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisA   1              20  (Event)
          0              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Constant      1.0912     0.8302     1.31  0.189
LogC           7.893      2.443     3.23  0.001
LogT           4.971      1.589     3.13  0.002

Log-Likelihood = -20.017
Test that all slopes are zero: G = 12.539, DF = 2, P-Value = 0.002

Goodness-of-Fit Tests

Method                Chi-Square    DF      P
Pearson                   30.048    27  0.312
Deviance                  34.489    27  0.152
Hosmer-Lemeshow           11.158     8  0.193

 Variance-Covariance Matrix of the Estimated Parameters

  0.68929   0.81403   0.19052
  0.81403   5.97053   3.73119
  0.19052   3.73119   2.52435
```

3.2 Miosis on Diameter Basis

Model D1: Binary Logistic Regression: MiosisD versus LogC, T, Sex, T*Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              14  (Event) → Pig 116 classified as having miosis.
          0              24
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Constant      13.627      4.444     3.07  0.002
LogC          18.509      5.995     3.09  0.002
T
  60           9.109      3.142     2.90  0.004
 180          13.484      4.481     3.01  0.003
Sex           0.8528     0.4973     1.71  0.086
T*Sex
  60          0.4567     0.7977     0.57  0.567
```

```
 180          1.1806      0.7522      1.57 0.117

Log-Likelihood = -12.729
Test that all slopes are zero: G = 24.559, DF = 6, P-Value = 0.000

Goodness-of-Fit Tests

Method                Chi-Square    DF       P
Pearson                   14.247    25   0.957
Deviance                  17.140    25   0.877
Hosmer-Lemeshow            6.467     8   0.595
```

Model D2: Binary Logistic Regression of MiosisD versus LogC, T, Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              13  (Event) → Pig 116 classified as NOT having miosis.
          0              25
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Constant      10.793      3.526      3.06  0.002
LogC          14.638      4.728      3.10  0.002
T
  60           7.077      2.523      2.80  0.005
 180          10.321      3.484      2.96  0.003
Sex           1.2820     0.4326      2.96  0.003

Log-Likelihood = -13.796
Test that all slopes are zero: G = 21.232, DF = 4, P-Value = 0.000

Goodness-of-Fit Tests

Method                Chi-Square    DF       P
Pearson                   21.879    27   0.743
Deviance                  22.047    27   0.735
Hosmer-Lemeshow           13.014     8   0.111
```

Model D3: Binary Logistic Regression of MiosisD versus LogC, T

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              13  (Event) → Pig 116 classified as NOT having miosis.
          0              25
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Constant       4.057      1.919      2.11  0.035
LogC           5.528      2.567      2.15  0.031
T
```

```
  60          2.276       1.512      1.51 0.132
 180          3.719       1.980      1.88 0.060

Log-Likelihood = -20.756
Test that all slopes are zero: G = 7.313, DF = 3, P-Value = 0.063

Goodness-of-Fit Tests

Method                Chi-Square    DF       P
Pearson                   29.435    26   0.292
Deviance                  33.194    26   0.157
Hosmer-Lemeshow            5.836     8   0.666
```

Model D4: Binary Logistic Regression of MiosisD versus LogC, LogT, Sex, LogT*Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              13  (Event) → Pig 116 classified as NOT having miosis.
          0              25
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Constant       3.079      1.295      2.38  0.017
LogC          17.225      5.754      2.99  0.003
LogT           9.749      3.415      2.85  0.004
Sex           -0.4140     0.9135    -0.45  0.650
LogT*Sex       1.1526     0.6206     1.86  0.063

Log-Likelihood = -12.349
Test that all slopes are zero: G = 24.127, DF = 4, P-Value = 0.000

Goodness-of-Fit Tests

Method                Chi-Square    DF       P
Pearson                   15.571    27   0.961
Deviance                  19.152    27   0.864
Hosmer-Lemeshow            4.224     8   0.836

Variance-Covariance Matrix of the Estimated Parameters

   1.6777    5.3187    2.6563   -0.0860    0.2962
   5.3187   33.1140   19.4085   -0.6041    1.7900
   2.6563   19.4085   11.6639   -0.3454    1.0326
  -0.0860   -0.6041   -0.3454    0.8345   -0.4937
   0.2962    1.7900    1.0326   -0.4937    0.3851
```

Model D5: Binary Logistic Regression of MiosisD versus Sex, LogC, LogT

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              13  (Event) → Pig 116 classified as NOT having miosis.
          0              25
```

```
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Constant       2.366      1.157     2.04  0.041
Sex           1.1713     0.4017     2.92  0.004
LogC          12.973      4.295     3.02  0.003
LogT           7.312      2.549     2.87  0.004

Log-Likelihood = -14.326
Test that all slopes are zero: G = 20.173, DF = 3, P-Value = 0.000

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
Pearson                    20.072    28  0.862
Deviance                   23.106    28  0.728
Hosmer-Lemeshow             7.669     8  0.466

 Variance-Covariance Matrix of the Estimated Parameters

   1.3391    0.2366    3.1653    1.4176
   0.2366    0.1614    1.2834    0.7365
   3.1653    1.2834   18.4432   10.7348
   1.4176    0.7365   10.7348    6.4972
```

Model D6: Binary Logistic Regression of MiosisD versus LogC, LogT

```
Link Function:  Normit

Response Information

Variable  Value       Count
MiosisD   1              13  (Event) ➔ Pig 116 classified as NOT having miosis.
          0              25
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Constant      1.0997     0.8118     1.35  0.176
LogC           5.583      2.343     2.38  0.017
LogT           2.992      1.474     2.03  0.042

Log-Likelihood = -20.757
Test that all slopes are zero: G = 7.310, DF = 2, P-Value = 0.026

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
Pearson                    29.525    27  0.336
Deviance                   33.196    27  0.191
Hosmer-Lemeshow             7.285     8  0.506

Variance-Covariance Matrix of the Estimated Parameters

  0.65901   0.81748   0.19815
  0.81748   5.48831   3.30769
  0.19815   3.30769   2.17183
```

4.0 LINEAR REGRESSION OF TRANSFORMED PUPIL SIZE ON LOGC, LOGT AND SEX

4.1 Pupil Area

Model ZA5: Regression Analysis of Z(area) versus Sex, LogC, LogT

Z is the Normit transform (the inverse of the cumulative distribution function of the standard normal distribution); area = minimum observed pupil area / baseline pupil area.
Sex coded -1 for female; 1 for male.

```
The regression equation is Z(area) = - 0.901 - 0.365 Sex - 4.47 LogC - 2.70 LogT

Predictor        Coef     SE Coef          T        P       VIF
Constant      -0.9009      0.4940      -1.82    0.077
Sex           -0.3646      0.1373      -2.65    0.012       1.1
LogC           -4.466       1.268      -3.52    0.001      10.2
LogT          -2.6965      0.8254      -3.27    0.002      10.1

S = 0.8060      R-Sq = 31.0%      R-Sq(adj) = 24.9%

Probit Slope = 4.466/0.8060 = 5.54

Toxic load exponent = 1.66; 95% Confidence Interval = (1.34,1.97)

Derived Probit Model:

Normit(fraction of pigs) = 1.11770 + 0.45234*Sex + 5.54036*LogC + 3.34554*LogT

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         3      9.9080      3.3027      5.08    0.005
Residual Error    34     22.0861      0.6496
Total             37     31.9941

Source       DF      Seq SS
Sex           1      1.8195
LogC          1      1.1563
LogT          1      6.9322

No evidence of lack of fit (P > 0.1)
```

Normplot of Residuals for Z(area)—the normal probability plot of the residuals is approximately a straight line, with all standardized residuals between -2 and 2.

Data Display – Note: this is the X'X-inverse matrix; multiply it by S-squared to get the Variance-Covariance matrix

```
 0.37571   0.01267   0.37233   0.05570
 0.01267   0.02903   0.08085   0.05047
 0.37233   0.08085   2.47681   1.53015
 0.05570   0.05047   1.53015   1.04890
```

4.2 Pupil Diameter

Model ZD5: Regression Analysis of Z(b) versus Sex, LogC, LogT
Z is the Normit transform (the inverse of the cumulative distribution function of the standard normal distribution). Sex coded -1 for female; 1 for male.

Note: b is the short axis of the pig's elliptical eye; in Z(b), b = short axis at minimum observed pupil area / average value of b for baseline images of the pig's eye.

```
The regression equation is
Z(b) = - 0.448 - 0.329 Sex - 4.21 LogC - 2.52 LogT

Predictor        Coef     SE Coef          T        P       VIF
Constant      -0.4485      0.4573      -0.98    0.334
Sex           -0.3292      0.1271      -2.59    0.014       1.1
LogC           -4.205       1.174      -3.58    0.001      10.2
LogT          -2.5160      0.7640      -3.29    0.002      10.1

S = 0.7460      R-Sq = 31.3%      R-Sq(adj) = 25.2%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         3      8.6070      2.8690      5.15    0.005
Residual Error    34     18.9228      0.5566
  Lack of Fit     28     14.8886      0.5317      0.79    0.695
  Pure Error       6      4.0341      0.6724
Total             37     27.5297

27 rows with no replicates

No evidence of lack of fit (P > 0.1)
```

Normplot of Residuals for Z(b)—the normal probability plot of the residuals is approximately a straight line, with all standardized residuals between –2 and 2.

Data Display – Note: this is the X'X-inverse matrix; multiply it by S-squared to get the Variance-Covariance matrix; this is the same as for Z(area) because the X's are the same

```
 0.37571   0.01267   0.37233   0.05570
 0.01267   0.02903   0.08085   0.05047
 0.37233   0.08085   2.47681   1.53015
 0.05570   0.05047   1.53015   1.04890
```

Data Display—Model coefficients

```
 -0.44847  -0.32923  -4.20524  -2.51600
```

5.0 REGRESSIONS INVOLVING TIME TO MIOTIC SIGNS

5.1 Regression of Time to Miosis on Exposure Concentration

Regression Analysis: Log(T50) versus LogC; Note: T50 = time to 50% of baseline area

```
The regression equation is
Log(T50) = 0.754 - 0.866 LogC
```

```
20 cases used 18 cases contain missing values


Predictor        Coef      SE Coef          T        P
Constant       0.7542       0.1259       5.99    0.000
LogC          -0.8664       0.1056      -8.21    0.000

S = 0.1628      R-Sq = 78.9%      R-Sq(adj) = 77.7%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         1      1.7858      1.7858     67.35    0.000
Residual Error    18      0.4773      0.0265
  Lack of Fit     15      0.4673      0.0312      9.34    0.045
  Pure Error       3      0.0100      0.0033
Total             19      2.2631

14 rows with no replicates

Unusual Observations
Obs       LogC   Log(T50)         Fit      SE Fit     Residual     St Resid
 37      -1.60     1.8249      2.1422      0.0607      -0.3173        -2.10R

R denotes an observation with a large standardized residual

No evidence of lack of fit (P > 0.1)
```

5.2 Regression of Time to Minimum Pupil Area on Exposure Duration, Exposure Concentration, and Gender

Regression Analysis: LogTmin versus Sex, LogC, LogT

```
The regression equation is
LogTmin = 0.984 + 0.0576 Sex + 0.408 LogC + 0.835 LogT

Predictor        Coef      SE Coef          T        P       VIF
Constant      0.98422      0.07356      13.38    0.000
Sex           0.05763      0.02045       2.82    0.008       1.1
LogC           0.4080       0.1889       2.16    0.038      10.2
LogT           0.8353       0.1229       6.80    0.000      10.1

S = 0.1200      R-Sq = 87.5%      R-Sq(adj) = 86.4%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         3      3.4155      1.1385     79.05    0.000
Residual Error    34      0.4897      0.0144
  Lack of Fit     28      0.4059      0.0145      1.04    0.533
  Pure Error       6      0.0838      0.0140
Total             37      3.9051

27 rows with no replicates

Lack of fit test
Possible interactions with variable Sex (P-Value = 0.002)
Possible interactions with variable LogC (P-Value = 0.001)
Possible interactions with variable LogT (P-Value = 0.001)
Overall lack of fit test is significant at P = 0.001
```

6.0 CALCULATION OF CONFIDENCE LIMITS FOR TOXIC LOAD EXPONENT

The binary logistic regression analysis routine in MINITAB® does not automatically provide confidence limits for toxic load exponents. The user must calculate these limits from other information provided by MINITAB®. To calculate the limits, values from the fitted model coefficients and the variance-covariance matrix are used in the following formula. Barry (1978) gives the standard error of a ratio, (α / β), which is based upon the propagation of error formula for a ratio:

$$\text{std err of } \left(\frac{\alpha}{\beta}\right) = \left(\frac{\alpha}{\beta}\right)\sqrt{\left(\frac{\text{var}(\alpha)}{\alpha^2}\right) + \left(\frac{\text{var}(\beta)}{\beta^2}\right) - (2)\left(\frac{\text{cov}(\alpha,\beta)}{\alpha\beta}\right)} \quad \text{[B1]}$$

where var(α), var(β), and cov(α, β) are the variance of the quantities, α and β, and their covariance, respectively. The 95% confidence limits for the ratio will equal $(\alpha / \beta) \pm (1.96)$(std err). For the case of the toxic load ratio, the ratio of interest is (k_C / k_T). Formula [B1] also applies to the toxic load exponent from models for pupil size, which were fit by Minitab's linear regression routine. The linear regression routine does not output the variance-covariance matrix of the estimated parameters, but rather a matrix, denoted $(X'X)^{-1}$, that must be multiplied by the square of the residual standard deviation to obtain the variance-covariance matrix of the estimated parameters.

7.0 REFERENCE

Barry, B.A. Errors in Practical Measurement Science, Engineering and Technology; John Wiley & Sons, Inc.: NY, 1978.